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Diversity of *Aspergillus* isolates and selection of an isolate with high β-fructofuranosidase activity that is native to the Peruvian coast

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In order to obtain a native isolate with high β-fructofuranosidase activity (FFase), a preliminary selection of 44 isolates, predominantly formed by *Aspergillus niger* (72%), was performed among 167 isolates of *Aspergillus* native to the Peruvian coast (Casa grande, Cartavio, Paramonga and Tacama). In addition, genetic diversity was studied using molecular markers, Inter Simple Sequence Repeats (ISSR). The FFase activity of these 44 isolates was compared and the isolate which showed the highest fructosyltransferase (FTase) activity was identified molecularly at the species level by DNA sequencing. Cluster analysis indicated 16 sub-genetic groups of which 11 of them were morphologically identified as *Aspergillus niger*. Samples from Paramonga showed the highest genetic diversity as explained by the Shannon diversity index (I= 0.21). No positive association was found between the genetic diversity of populations and FFase activity. Isolates PR-151, PR-144 and PR-142 showed the highest FFase activity in 5 consecutive evaluation generations, PR-142 being the most active with a total FFase activity of 11,248 ($U.L^{-1}$) and 6.17 g/L of biomass indicating a great biotechnological potential for the synthesis of prebiotics. The molecular identification at the species level confirmed that PR-142 belongs to *A. niger* lineage.

Key words: β-Fructofuranosidase, fructosyltransferase, *Aspergillus*, Inter Simple Sequence Repeat (ISSR), fructooligosaccharides.

INTRODUCTION

The *Aspergillus* species belong to the group of filamentous fungi that are widely disseminated in the environment, some of which are associated with mycotic diseases while others are of great economic importance.

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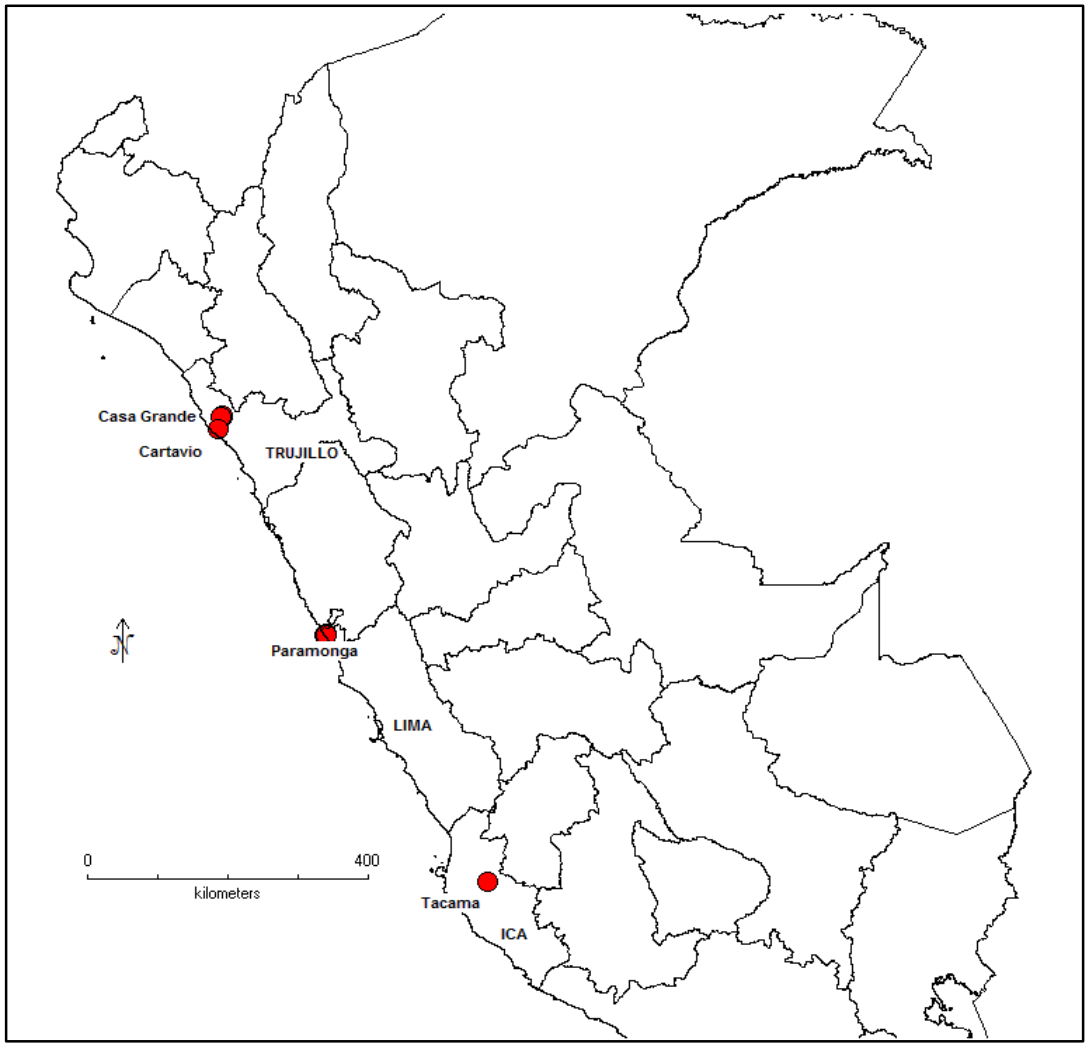

Figure 1. Map showing the locations of the four *Aspergillus* populations used in the present study.

Thus, these are used in the production of amylases for the production of biofuels (Zheng et al., 2012), or in the synthesis of metallic nanoparticles that are used in medicine and electronics (Juraifani and Ghazwani, 2015). In the pharmaceutical and cosmetic industries, it has been found that they can be used as an acidulant and dissolving agent of active ingredients (Pau et al., 2015). In the functional food industry, fructooligosaccharides (FOS) are produced by enzymatic synthesis catalyzed by microbial enzymes, such as those produced by species of the *Aspergillus* genus. FOS is highly valued for its beneficial properties, such as selective increases in beneficial gut bacteria (bifidobacteria), prevention of colon cancer, reduction of cholesterol levels and triglycerides in the blood, and can be considered as a calorie-free and non-cryogenic sweetener (Maiorano et al., 2008). FOS-producing enzymes are classified as -fructofuranosidases (FFase, EC 3.2.1.26) with high fructosyltransferase activity (FTase, EC 2.4.1.9) which in the case of *Aspergillus*, as in other microorganisms, both activities are performed by a single enzyme. The study of the genetic diversity of *Aspergillus* with respect to biotechnological capacity in Peru has implications for the creation of local bio-industries.

MATERIALS AND METHODS

Sampling and isolation of *Aspergillus*

Soil samples were taken from sugarcane fields in 4 coastal regions of Peru: North Coast (Casa Grande and Cartavio), Central Coast (Paramonga), and South Coast (Tacama) (Figure 1). From a series of dilutions, filamentous fungi were isolated using widely-used microbiological techniques with Czpeck medium. Monosporic cultures were preserved in 10% glycerol and stored at -80°C. Using microscopic techniques, only *Aspergillus* species were identified based on the typical form of the conidiophore, the presence of filiades and the standing cells that are attributed to this genus (Pitt and Hocking, 1985).

Morphological identification

Identification of the species was performed in Czapeck solid medium using the adhesive tape methodology. Morphological identification was performed using macro and microscopic features following the taxonomic keys of Thom and Raper (1945) and Watanabe (2002).

Molecular identification

To enable molecular identification, genomic DNA extraction was performed using the methodology developed by Liu et al. (2000). The DNA was amplified using the following primers: The ITS1 universal primer (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White et al., 1990). The primers Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3´) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3´) of the partial sequence of the β-tubulin gene (Glass and Donaldson, 1995) and the primers Cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and Cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) of the calmodulin gene (Hong et al., 2006). The sequences obtained were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov).

Preliminary selection

The preliminary selection included the evaluation of the isolates according to their ability to hydrolyze sucrose (invertase activity or FFase), using the methodology described by Guilarte et al. (2009) which consists of taking circular portions of 7 mm mycelium grown in potato dextrose agar (PDA) as a source of enzyme. The enzymatic reaction was in 50 ml conical tubes with 1.2 ml of 0.2 M tris-acetate buffer at pH 5.5, using 3.7 ml of 64% (w/v) sucrose as a substrate. The control consisted of circular fragments of the same medium but without mycelium. Triplicate tubes were placed in a shaking water bath GFL-1083 (Biovendis Ltd.) at 100 rpm for 60 min at a temperature of 50°C. The amount of released reducing sugars was quantified by the 3.5-Dinitrosalicylic acid (DNS) method (Miller, 1959), while considering the mean absorbance values (OD 540 nm) as the selection factor (Table 1). Subsequently, the isolates that presented the highest values were evaluated in the same way for 5 consecutive generations in order to eliminate possible errors and to verify the genetic stability of the isolates so as to maintain the FFase activity.

FFase activity assay in shaken flasks

With the most representative isolates from the previous stage, FFase activity assays were performed. For this purpose, 10^7 spores per ml^{-1} were cultured in 250 ml flasks containing 50 ml of fermentative medium whose composition was (g/L): 3.0 sucrose, 0.3 $NaNO_3$, 0.2 KH_2PO_4, 0.05 $MgSO_4.7H_2O$, 0.02 $MnCl_2.4H_2O$, and 0.001 $FeSO_4.7H_2O$, with pH of 5.5. These were incubated on a rotary shaker at 30°C and 200 rpm for 72 h. The mycelia were obtained using a polysulfone (PSF) filtration kit (Nalgene Ltd.) and Whatman ® quantitative filter paper grade Nº 1 in a vacuum and then washed 3 times with distilled water. The FFase activity of the mycelia was obtained using approximately 0.02 g mass of enzyme source, while the extracellular activity was performed with 0.3 ml of the fermentation broth. The FFase activity linked to mycelium was defined as that catalysing the formation of 1 µmol reducing sugar per minute under the above conditions.

FTase activity test

With the most active isolate selected in the previous steps, the FTase activity linked to mycelium and extracellular activities was determined. For this purpose, the above-described methodology was used. The reaction products were analyzed by liquid chromatography with refractive index detector (LC-RID) on an Agilent Technologies 1220 Infinity LC system-1260 RID equipment (Boeblingen, Germany). Separation was performed on a HPLC column Kromasil® (100-NH_2) (Akzo Nobel, Brewster, NY, USA) (250 x 4.6 mm i.d., 5 µm particle size) using 70:30 (v/v) acetonitrile/water as the mobile phase and isocratic elution with a flow rate of 1.0 ml min^{-1} for 30 min. One unit of fructosyltransferase activity was defined as the amount of enzyme producing 1 µmole of FOS (1-kestose plus nystose) per min, under the assay conditions.

Molecular diversity based on Inter Simple Sequence Repeat - Random Amplified Polymorphic DNA (ISSR-RAPD) markers

Genomic DNA extraction was performed using the methodology developed by Liu et al. (2000) for filamentous fungi. The PCR reaction was undertaken in a Mastercycler® Nexus Gradient Thermal Cycler (Eppendorf, Germany) using 6 polymorphic primers (Table 4). The thermal amplification conditions as well as the concentrations of each component of the PCR were taken from Neal et al. (2011). The PCR products were resolved on 1.5% agarose gels and stained with 0.5 mg mL^{-1} ethidium bromide. The inter simple sequence repeat (ISSR) band patterns were assigned values of 1 (band presence) and 0 (band absence). An array of similarities was constructed using the Jaccard Similarity Index from which the dendrogram was constructed by using the neighbor-joining or UPGMA method with the NTSYS-PC program version 2.0. The reliability of the dendogram was verified by performing a cophenetic correlation analysis derived from the Mantel test using the same program. For the genetic analysis, populations were subdivided in two ways by their geographical origin (4 populations) and by the value of the absorbance reached in three levels: High (OD 540 > 1), medium (0.5 <OD540 < 1), and low (OD540 < 0.5). Genetic diversity was estimated from the Shannon Indices (I), from the average heterozygosity (h), genetic differentiation between populations (GST), as well as genetic flow (Nm) by using the POPGENE program version 1.32. The distribution of diversity among populations and within populations was calculated using the analysis of molecular variance (AMOVA) within the GENALEX 6.5 program.

RESULTS

Preliminary selection

From a total of 167 *Aspergillus* lineages, only 44 isolates showed a higher capacity for invertase activity (Table 1) than the control and were grouped according to their optical density (OD) at 540 nm as follows: High: 9 isolates; median: 14 isolates; and low: 21 isolates. The evaluation of the 9 isolates in the 5 consecutive generations (Figure 2) showed that isolates PR-142, PR-152 and PR-144 presented the highest values of absorbance and were the only isolates that were considered for the next stage of analysis in shaking flasks.

Identification of *Aspergillus* species

Following the taxonomic keys, 32 isolates were identified

Table 1. List of the 44 isolates with invertase activity measured at 540nm and their species identification.

Code	Species	Origin	OD_{540}	Level
PR-144	*A. niger*	Paramonga	1.92 ± 0.07	High
CR-101	*A. niger*	Cartavio	1.76 ± 0.03	High
TA-124	*A. niger*	Tacama	1.73 ± 0.05	High
CR-98	*A. niger*	Cartavio	1.65 ± 0.04	High
TA-116	*A. niger*	Tacama	1.40 ± 0.03	High
PR-151	*A. niger*	Paramonga	1.36 ± 0.01	High
CR-110	*A. niger*	Cartavio	1.34 ± 0.03	High
TA-122	*A. niger*	Tacama	1.09 ± 0.06	High
PR-142	*A. niger*	Paramonga	1.08 ± 0.05	High
PR-150	*A. niger*	Paramonga	0.75 ± 0.08	Medium
TA-114	*A. niger*	Tacama	0.74 ± 0.05	Medium
PR-148	*A. niger*	Paramonga	0.73 ± 0.03	Medium
CG-59	*A. niger*	Casa Grande	0.71 ± 0.02	Medium
CR-96	*A. niger*	Cartavio	0.63 ± 0.03	Medium
CG-30	*A. niger*	Casa Grande	0.62 ± 0.02	Medium
TA-126	*A. niger*	Tacama	0.62 ± 0.06	Medium
CG-29	*A. niger*	Casa Grande	0.62 ± 0.06	Medium
CG-57	*A. niger*	Casa Grande	0.61 ± 0.01	Medium
CR-87	*A. niger*	Cartavio	0.6 ± 0.03	Medium
CR-91	*A. niger*	Cartavio	0.6 ± 1.20	Medium
CR-90	*A. niger*	Cartavio	0.54 ± 0.05	Medium
CG-40	*A. niger*	Casa Grande	0.60 ± 0.04	Medium
CG-64	*A. niger*	Casa Grande	0.53 ± 0.07	Medium
PR-143	*A. niger*	Paramonga	0.44 ± 0.01	Low
CR-109	*A. niger*	Cartavio	0.13 ± 0.03	Low
CR-89	*A. niger*	Cartavio	0.39 ± 0.01	Low
CR-100	*A. niger*	Cartavio	0.38 ± 0.04	Low
CR-107	*A. fumigatus*	Cartavio	0.33 ± 0.01	Low
CG-50	*A. niger*	Casa Grande	0.33 ± 0.02	Low
CG-106	*A. flavus*	Casa Grande	0.31 ± 0.04	Low
CG-58	*A. niger*	Casa Grande	0.30 ± 0.02	Low
CG-47	*A. niger*	Casa Grande	0.25 ± 0.02	Low
CR-86	*A. versicolor*	Cartavio	0.25 ± 0.03	Low
TA-115	*A. ochraceus*	Tacama	0.21 ± 0.08	Low
CR-106	*A. flavus*	Cartavio	0.31 ± 0.04	Low
CG-103	*A. flavus*	Casa Grande	0.15 ± 0.09	Low
TA-119	*A. niger*	Tacama	0.13 ± 0.06	Low
PR-141	*A. fumigatus*	Paramonga	0.21 ± 0.03	Low
PR-147	*A. fumigatus*	Paramonga	0.12 ± 0.04	Low
CR-108	*A. fumigatus*	Cartavio	0.12 ± 0.04	Low
CG-104	*A. flavus*	Casa Grande	0.13 ± 0.08	Low
CG-39	*A. niger*	Casa Grande	0.10 ± 0.03	Low
PR-146	*A. fumigatus*	Paramonga	0.09 ± 0.05	Low
PR-154	*A. fumigatus*	Paramonga	0.08 ± 0.03	Low

as *A. niger*, 6 isolates were identified as *A. fumigatus*, 1 isolate was identified as *A. versicolor*, 1 isolate was identified as *A. ochraceus*, and 4 isolates were identified as *A. flavus* (Table 1).

Determination of FFase activity

The native PR-142 isolate showed the highest value of mycelial and extracellular FFase activities, having a total

Table 2. Parental mycelial and extracellular FFase activities of the 3 most representative native isolates.

Isolate	Intracellular activity (U.L^{-1})	Extracellular activity (U.L $^{-1}$)	Total activity (U.L^{-1})	Biomass (g/L^{-1})
PR-151	1,066.7 ± 179.3^{b}	6,350.00 ± 23^{ab}	7416.7	3.17 ± 0.55^{c}
PR-144	112.4 ± 58.3^{c}	4,140.00 ± 80^{b}	4252.4	4.47 ± 1.12^{b}
PR-142	3,528.2 ± 49.8^{a}	7,720.00 ± 77^{a}	11248.2	6.17 ± 0.74^{a}

[1]Mean of six samples (n=6) ± SD. The different letters indicate significant differences ($p < 0.05$).

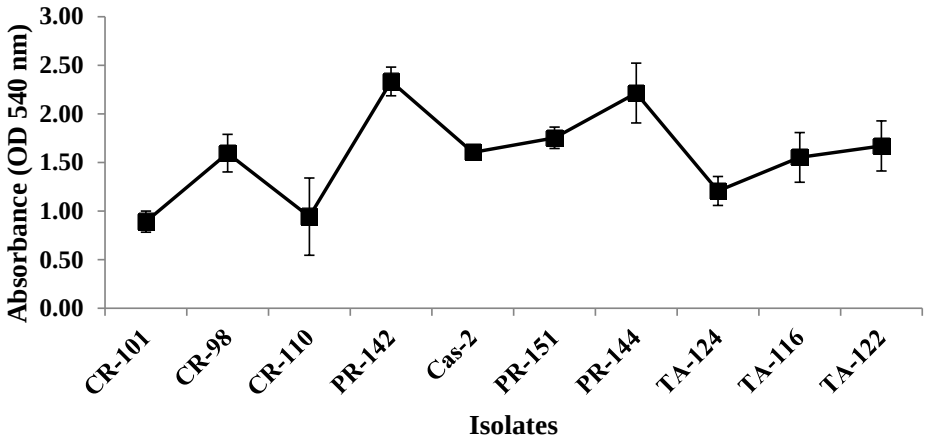

Figure 2. Average absorbance values (3 replicates) in 5 consecutive generations of 9 *Aspergillus* isolates.

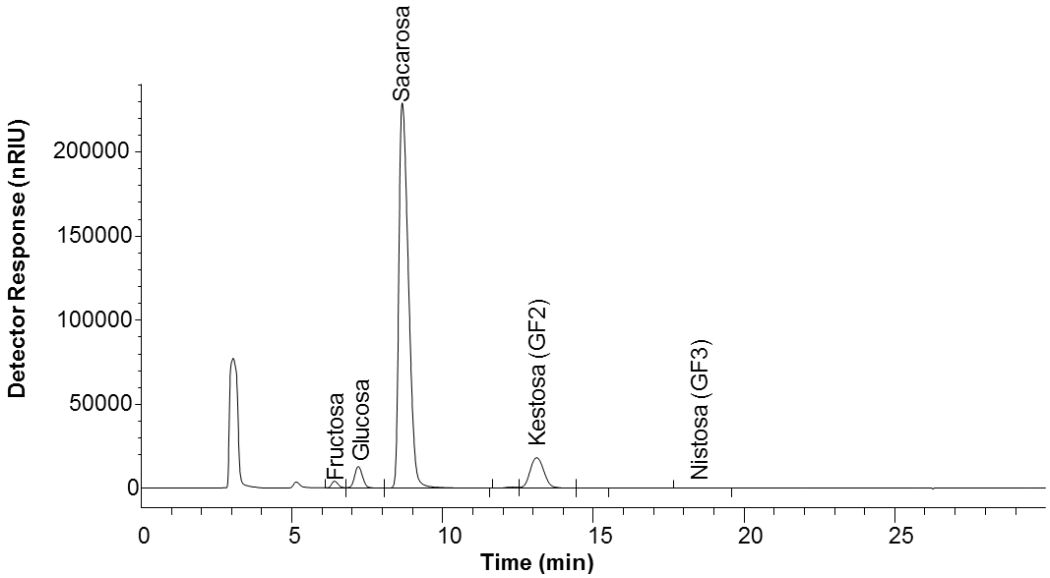

Figure 3. LC-RID profile after 72 h of fructosyltransferase reaction catalyzed by PR-142 isolate.

activity that represents 1.5 times the activity of the PR-144 isolate. It also presents the highest biomass among all the isolates and was therefore considered the most representative (Table 2).

Determining of FTase activity

LC-RID analysis of isolate PR-142 yielded a typical FOS chromatogram (Figure 3) consisting of 86.6% GF2 (1-

Table 3. GenBank accession numbers and similarity of ITS region, calmodulin and β-tubulin gene sequences of PR-142.

Gene	Closest species	GenBank accession number	Similarity (%)
	Aspergillus welwitschiae	KR020702.1	100.0
	A. niger	KM593202.1	100.0
	Aspergillus niger	KX244952.1	100.0
ITS	Fungal sp. isolate Xmf163	KX098122.1	100.0
	A. niger	KX664417.1	100.0
	A.welwitschiae	KU711861.1	97.0
β-tubulin (BT2)	*A. niger*	KT965691.1	97.0
	A. niger	HQ632739.1	97.0

Table 4. Characterization of the 6 primers used in the ISSR analysis.

Initiator	Secuence 5´→3´	Polymorphic bands	PIC
ISSR1	(5'-GTGGTGGTGGTGGTG-3')	11	0.19
ISSR2	(5'-GACAGACAGACAGACA-3')	14	0.10
UBC817	(5-'CACACACACACACACAA-3')	12	0.23
UBC834-C	(5'-AGAGAGAGAGAGAGAGCT-3´)	18	0.16
UBC809	(5'-AGAGAGAGAGAGAGAGG-3')	20	0.22
UBC895	(5´AGAGTTGGTAGCTCTTGATC-3´)	13	0.18
Total		88	

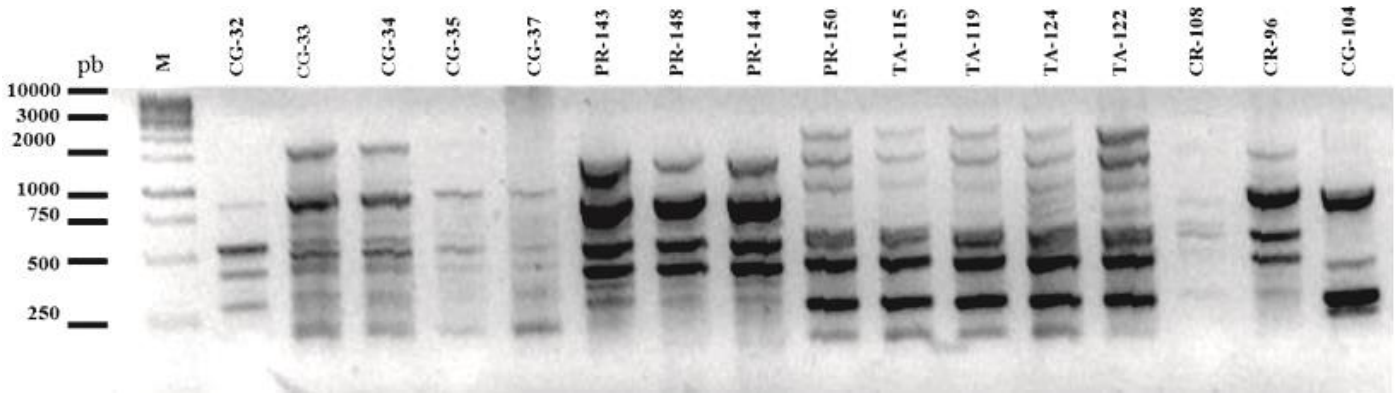

Figure 4. ISSR band patterns generated from some Aspergillus isolates using primer UBC-834. Gene Ruler 1 kb DNA ladder (M) (numbers represent size in bp).

kestose) and 13.4% GF3 (1-nystose). FOS production was 18.4 g/L, mycelial activity was 11,500 ($U.L^{-1}$), and extracellular activity was 7400 $U.L^{-1}$ ($p<0.05$). The range of fructosyltransferase and hydrolytic activities (At/Ah) was 1.8.

Molecular characterization

Molecular analysis of the PR-142 isolate showed that it is an *Aspergillus* isolate related to the *A. niger* as a 100% similarity with the 5.8S-ITS region, 97% similarity with the partial sequence of the β-tubulin gene, and 97% similarity with the sequence of the calmodulin gene were observed (Table 3).

ISSR analysis

Of the 10 initiators initially employed, only 6 gave clear bands that were used in the analyses (Table 4). With these primers, a total of 88 bands were generated, of which 81 (92.05%) were polymorphic (Figure 4). The sizes of the fragments chosen for analysis were from 100

Table 5. Measures of genetic diversity of 44 isolates of *Aspergillus* according to their geographic origin.

Population	N	PL%	h	I
Casa Grande	13	46.59	0.1253 ± 0.173	0.1967 ± 0.249
Cartavio	14	47.73	0.1120 ± 0.153	0.1818 ± 0.228
Paramonga	10	55.68	0.1252 ± 0.142	0.2082 ± 0.216
Tacama	7	40.91	0.1167 ± 0.170	0.1820 ± 0248

Total population GST: 0.135; Nm: 3.18%; PL: Percentage of polymorphic loci; N: Number of isolates. h: Genetic diversity of Nei (1973); I: Shannon Diversity Index (Lewontin, 1972).

to 2000 bp. The first, UBC 817 and UBC834, were those that generated the highest polymorphism indexes (polymorphism information content, PIC values) (Table 4). Analysis of the grouping (Figure 5) allowed us to separate 6 groups, one of them being composed of *A. niger* (Group A) and another being composed of other species (B, C, D, E and F). The *A. niger* formed 11 sub-groups (32 isolates). The isolates belonging to other species formed 5 subgroups: Group XII was formed of two *A. fumigatus* isolates (PR147 and PR154), Group XIII was formed of two isolates of *A. flavus* (CG103 and CG104), Group XIV was formed of the CG106 isolates of *A. flavus*, Group XV was formed of the CR86 isolates of *A. versicolor* and the TA115 isolates of *A. ochraceus*, and Group XVI was formed of 6 *A. fumigatus* isolates (CR106, CR108, PR146, CR107, PR141). It was determined that pairs of isolates CG29/CG30, CG64/CR87, CR106/CR108 are duplicates. The polymorphism in the evaluated populations ranged from 40.91 to 55.68% (Table 5). The Paramonga population presented the greatest polymorphism among all populations, while the Tacama population had the lowest polymorphism. Genetic diversity (h) in these populations ranged from 0.11 (Cartavio) to 0.12 (Casa Grande), the latter being similar to the Paramonga population (0.12) (Table 5). The Shannon Index (I) presented very similar values and was in the range of 0.18 to 0.20, being in the locality of Paramonga which presented a greater value (0.20). The genetic diversity attributable to genetic differentiation between populations (Gst) was 0.13 on all loci, hence a low differentiation of these populations could be considered as having similar results to the obtained AMOVA ($p<0.001$) which attributed 80% all variation between isolates within populations and only 20% between different populations. The populations of Cartavio and Paramonga were genetically the closest (0.02), while the most distant populations were those of Tacama and Casa Grande (Figure 6). Gene flow among these populations was low (Nm = 0.13) among these 4 populations. The cophenetic correlation between similarity matrix and the dendrogram showed a good fit with a correlation coefficient (r) of 0.95.

DISCUSSION

Native isolates of *A. niger* that were isolated from the three coastal regions of Peru showed potential for biotechnological use in the fruit industry including the production of fructooligosacharides (FOS). The PR-142 isolate had a fairly similar mycelial activity as compared to another native isolate, *Aspergillus oryzae* IPT-301 (Cuervo et al., 2007) which exhibited a mycelial activity value of 3,599 (UL^{-1}). However, the extracellular activity of PR-142 isolate was lower than that of IPT-301, whose reported value was 14,190 (UL^{-1}) and the ratio of At/Ah was also lower than that of IPT-301 which had an activity value of 5.2. This means that this isolate has a lower rate of FOS formation (Cuervo et al., 2007). These innate characteristics of the native enzyme can be enhanced by mutagenesis techniques (Guilarte et al., 2009) or recombinant DNA technologies (Zhang et al., 2015). The FFase capacity of *A. niger* has been known for a long time and is used in the production of FOS (Maiorano et al., 2008). However, there are few reports on FOS synthesis capacity of *A. flavus* (Praveen et al., 2010), *A. versicolor* (Belorkar et al., 2015) and *A. ochraceus* (Guimarães et al., 2007). It is noteworthy that the same microorganism can produce different enzymes with different characteristics via transfructosylation activity (Yoshikawa et al., 2007).

This study has shown that it is possible to know the genetic diversity of 44 isolates of *Aspergillus* having the ability to hydrolyze sucrose through the use of ISSR markers. The efficiency of ISSR markers in the study of *Aspergillus* diversity has been evidenced in different reports (Zhang et al., 2013; Neal et al., 2011). The current results show poor overall diversity and low genetic differentiation which would demonstrate these populations are closely related despite the geographic distance that separates them. Similar results were obtained in black *Aspergillus* originating in 6 European countries (Perrone et al., 2006). In addition, no relationship between the high FFase activity shown by the strains from *A. niger* and their geographical origin was found, which could be attributable to the fact that these microorganisms are derived from soils intensively used in the production of sugar cane. Similar results were reported by using other *Aspergillus* strains with high capacity of synthesizing FOS which was also isolated from this type of soils (Aziani et al., 2012; Cuervo et al., 2007). The ISSR dendrogram (Figure 5) did not provide any correlation to the hydrolytic capacity due to the most

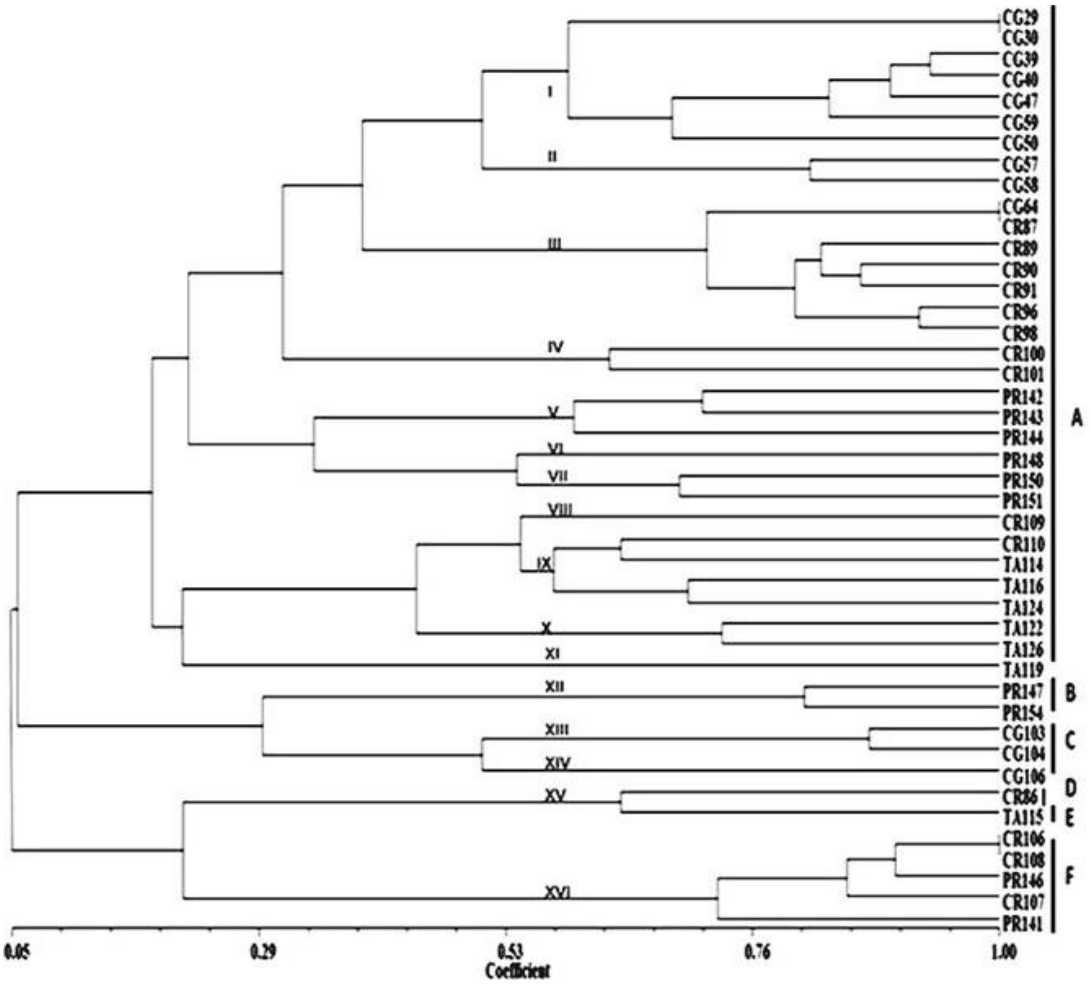

Figure 5. Dendogram obtained from 44 isolates with FFase activity using the Jaccard coefficient and the UPGMA algorithm. The vertical lines on the right-hand side indicate the formation of 14 genetic groups with a coefficient of 0.53.

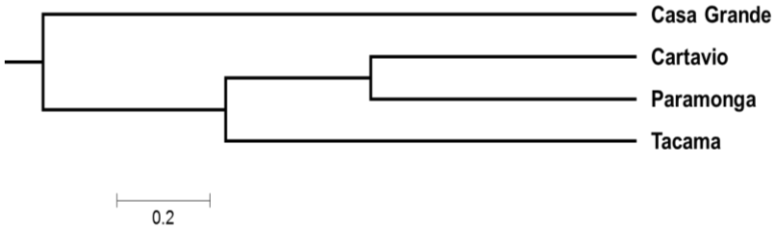

Figure 6. Dendogram based on the genetic distance of Nei (1973), showing the relationship between the four geographic populations of *Aspergillus*. The bar scale denotes genetic distance.

active strains were grouped in different genetic groups. This behaviour could indicate that the ISSR fragments are not included in the genes associated with sucrose metabolism. Similar data in *A. parasiticus* and *A. terreus* were reported by Juraifani and Ghazwani (2015) who also used ISSR to identify potential microorganisms with ability of producing silver nanoparticles (AgNPs).

On the other hand, the genetic diversity observed in populations categorized according to their hydrolysis capacity had even lower Gst and AMOVA indexes, stressing, thus, the inadequacy of classifying populations based on their hydrolytic capacity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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